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PRINCIPAL INVESTIGATOR: Kathryn B. Horwitz, Ph.D.

CONTRACTING ORGANIZATION:

University of Colorado Health Sciences Center
Division of Endocrinology, Metabolism and Diabetes
4200 E. 9th Avenue, Denver, CO 80262

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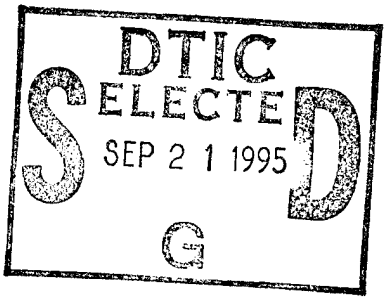

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13. ABSTRACT (Maximum 200 words)

Progesterone promotes the development and growth of breast cancers. Inhibitors of progesterone are in clinical trials for the treatment of breast cancers. A problem with all endocrine therapies is that the tumors eventually become resistant. We will study the molecular biology of antiprogestins in breast cancer, focusing on mechanisms by which their inhibitory actions are inappropriately switched to stimulatory ones. Antiprogestins regulate gene transcription by binding to progesterone receptors (PR). Human breast cancer cells contain mixtures of two PR -- truncated A-receptors, and B-receptors that have an N-terminal extension (the B-upstream segment, BUS). A- and B-receptors are functionally dissimilar, and antagonist-occupied B-receptors (but not A-receptors) stimulate transcription inappropriately. Aim 1 is to construct new breast cancer cell lines that contain only A- or B-receptors. The independent transcriptional function of each receptor will be tested, and conditions for the inappropriate activation by progestins will be defined. Aim 2 will focus on BUS: its interaction with the rest of the PR molecule; its activation by cAMP; and its activation by phosphorylation. In Aim 3 we will identify, isolate and clone cDNAs for coactivator transcription factors that uniquely interact with BUS and select the direction of B-receptor transcription. These studies will define the molecular mechanisms of progesterone action in breast cancer, and the means by which tumors become hormone-resistant.

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FOREWORD

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K B Harris 7/1/95

PI - Signature Date

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INTRODUCTION

Nature of the Problem and Background:

Women develop breast cancer because they have ovaries. Analyses of factors involved in breast cancer induction, growth, and treatment has therefore focused on estradiol and progesterone; particularly on estradiol. The latter is a growth promoter, and blockade of estradiol at the tumor with the antiestrogen tamoxifen is a major thrust of current endocrine therapies. But is estradiol the only ovarian hormone with an impact on breast cancer development and proliferation? Progesterone has a proliferative effect in the epithelium of the normal breast, and progestin agonists at physiological doses promote the growth of experimental mammary cancers. Progesterone antagonists inhibit the growth of breast cancer (1-3).

In human breast cancers there are two, naturally occurring, PR isoforms: the 769 amino acid A-receptors; and the 933 amino acid B-receptors, that have a 164 amino acid extension at the N-terminus -- which we call the B-upstream segment, or BUS. Downstream of BUS, A- and B-receptors are identical. The two receptors can form homo- and heterodimers, leading to three dimeric species (A:A, A:B, B:B) that bind DNA (1, and references therein). Our preliminary data show that the A- and B-isoforms are functionally dissimilar when occupied by agonists. Additional data show that progesterone antagonists can either inhibit or stimulate transcription, depending on the receptor isoform, the promoter of the gene being regulated, and the modulatory influence of other signalling pathways, particularly of cAMP (4,5). We postulate that breast cancers become "resistant" to hormone therapy because antagonists acquire inappropriate, agonist-like, effects. The clinical consequences of such a functional switch are grave. The studies we have proposed address the molecular mechanism by which antagonist-occupied progesterone B-receptors become transcriptional agonists.

Purpose of Present Work and Methods of Approach:

Aim 1. The functional differences between A- and B-receptors in breast cancer cells. B- and A-receptors are present together in breast cancers. Transient transfection methods show that antagonist-occupied B-receptors, but not A-receptors, can act like transcriptional agonists in a promoter-specific manner or when cAMP levels are increased. We plan to stably transfect PR-negative breast cancer cell lines with expression vectors encoding B- or A-receptors. This allows each receptor isoform to be studied independently while being expressed at normal levels in a physiological setting. The cells will be used to assess long-term growth effects, and transcriptional regulation, by progesterone agonists and antagonists, with or without increased cAMP levels. For transcriptional studies, different promoter-reporter constructs will be analyzed. These studies will define the effects of each receptor isoform on the functional end-points of transcription and cell proliferation in the physiologically relevant setting of breast cells.

Aim 2. BUS - The B-upstream segment. A third transcriptional activation domain unique to B-receptors? Since only PR B-receptors anomalously induce transcription in the presence of progesterone antagonists, we will focus on their unique 164 amino acid extension -- the B-

upstream segment, or BUS. Preliminary data show that BUS contains a novel transcriptional activation function we call AF-3. This is in addition to two other AFs (AF-1 and AF-2) that are common to both receptor isoforms. We postulate that AF-3 functions by binding one or more coactivator proteins. We will construct a series of expression vectors of BUS alone, or of BUS fused to the DNA binding domain (DBD) and nuclear localization signal (NLS) of PR. These constructs will be tested for their ability to constitutively regulate transcription; to "squelch" full-length B-receptor actions; to complement A-receptors; and to be cAMP modulated, all in promoter and cell-specific contexts. Mutants will be constructed of 5 *ser-pro* clusters found on BUS, in order to map AF-3, and to analyze the control of B-specific transcription by phosphorylation. The studies in this aim will functionally define and characterize AF-3; a site unique to B-receptors.

Aim 3. Mechanisms of AF-3 action in the BUS segment. Antagonist-occupied B-receptors activate transcription in a promoter-specific fashion: on the mouse mammary tumor virus (MMTV) promoter when cAMP levels are elevated; on the *Herpes simplex* virus thymidine kinase (*tk*) promoter, through a novel, PRE-independent mechanism. First, we plan to identify *cis*-acting elements on the MMTV and *tk* promoters through which antagonist-occupied B-receptors stimulate transcription. Site-specific mutants of the MMTV promoter will seek the *cis*-acting elements that eliminate cAMP effects without loss of PR-regulated transcription. We will test the hypothesis that cAMP acts through novel DNA elements that cooperatively bind the ATF/CREB and HMG family of proteins, and interact with BUS. Linker-scanning mutants of the *tk* promoter will be used to define elements that mediate antagonist-occupied B-receptor stimulation of transcription. Second, protein-protein interactions between antagonist-occupied B-receptors and as yet unknown coactivators will be characterized using bacterially produced BUS fusion proteins, or the yeast two-hybrid system to identify, isolate and clone cDNAs encoding nuclear proteins that interact with BUS and to analyze their tissue-specific distribution. The studies in this aim will define novel coactivator proteins that interact with B receptor isoform of PR, and select their direction of transcription.

BODY

Two papers related to these aims have been published:

a) Sartorius CA, Groshong SD, Miller LA, Powell RL, Tung L, Takimoto GS and Horwitz KB. New T47D breast cancer cell lines for the independent study of progesterone B- and A-receptors: only B-receptors are switched to transcriptional agonists by antiprogestins plus cAMP. CANCER RESEARCH, 54:3868-3877, 1994.

Because progesterone antagonists are growth inhibitors, they are in phase III clinical trials for the treatment of breast cancers. However, when cellular cAMP levels are elevated, some antiprogestins inappropriately activate transcription. We have proposed that hormone "resistance" may result from such unintended stimulation of breast cancers by antagonists. In transient expression systems, the two natural isoforms of human progesterone receptors (PR), B-receptors and truncated A-receptors, have dissimilar effects on agonist-mediated transcription. We have shown that in the presence of 8 Br cAMP, antiprogestin-occupied B-receptors but not A-receptors become transcriptional activators. Therefore, as proposed in Aim 1, we developed new model systems to study each PR isoform independently in a breast cancer setting: (a) a stable PR-negative monoclonal subline (T47D-Y) of PR-positive T47D breast cancer cells was selected by flow-cytometric PR screening. T47D-Y cells are PR-negative immunologically; by ligand binding assay; by growth resistance to progestins; by failure to bind a progesterone response element (PRE) *in vitro*; and by failure to transactivate PRE-regulated promoters; and (b) T47D-Y cells were stably transfected with expression vectors encoding one or the other PR isoform, and two monoclonal cell lines were selected that express either B-receptors (T47D-YB) or A-receptors (T47D-YA) at levels equal to those seen in natural T47D cells. The ectopically expressed receptors are properly phosphorylated, and like endogenously expressed receptors, they undergo ligand-dependent down-regulation. The expected B:B or A:A homodimers are present in cell extracts from each cell line, but A:B heterodimers are missing in both. In the presence of agonists, cAMP-dependent, transcriptional synergism of PRE-regulated promoters is seen in both cell lines. By contrast, in the presence of the antiprogestins RU486 or ZK112993, inappropriate transactivation occurs in YB cells but not in YA cells. The class of antiprogestins represented by ZK98299, which blocks PR binding to DNA, does not activate transcription in either cell line.

We propose that these new cell lines are physiological models for the study of PR isoform-specific antiprogestin resistance in breast cancer. These cells will next be used to study isoform-specific growth regulatory mechanisms of progestins and antiprogestins to complete Aim 1.

b) Sartorius CA, Melville MY, Hovland AR, Tung L, Takimoto GS and Horwitz KB. A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. MOLEC ENDOCRINOL, 8:1347-1360, 1994.

As described above, the two PR isoforms differ functionally when they are occupied by agonists or antagonists. We postulated that BUS is in part responsible for the functional differences between the two isoforms. As proposed in Aim 2, we have constructed a series of

CONCLUSIONS

The completed research has created novel model systems with which to further study the two forms of PR. We have also defined an important transcriptional activation domain in PR that we believe accounts for the functional differences between the two receptors at a molecular level. Future work is as outlined in the grant application. No changes in direction are foreseen at this juncture.

hPR expression vectors encoding BUS fused to isolated downstream functional domains of the receptors. These include the two transactivation domains, AF1 located in a 90 amino acid segment just upstream of the DNA binding domain (DBD) and nuclear localization signal (NLS); and AF2 located in the hormone binding domain (HBD). BUS is a highly phosphorylated domain, and contains the serine residues responsible for the hPR_B triplet protein structure. The construct containing BUS-DBD-NLS binds tightly to DNA when aided by accessory nuclear factors. In HeLa cells, BUS-DBD-NLS strongly and autonomously activates transcription of chloramphenicol acetyl transferase (CAT) from a promoter containing two progesterone response elements (PRE_2-$TATA_{tk}$-CAT). Transcription levels with BUS-DBD-NLS are equivalent to those seen with full-length hPR_B, and are higher than those seen with hPR_A. BUS specifically requires an intact hPR DBD in order to be transcriptionally active. DBD mutants that can not bind DNA, or whose DNA binding specificity have been switched to an estrogen response element, cannot cooperate in BUS transcriptional activity. The function of BUS-DBD-NLS is promoter and cell-specific. It does not transactivate a CAT reporter driven by the mouse mammary tumor virus promoter in HeLa cells, and poorly transactivates PRE_2-$TATA_{tk}$-CAT in PR-negative T47D breast cancer cells. However, in the breast cancer cells, BUS-DBD-NLS transactivation of PRE_2-$TATA_{tk}$-CAT can be reconstituted either by elevating cellular levels of cAMP or by linking BUS and DBD to AF1 or AF2 of hPR, each of which alone is also inactive in these cells.

We conclude that hPR_B contain a unique third activation function (AF3) located within BUS and requiring the functional DBD of hPR. Depending on the promoter or cell tested, AF3 can activate transcription autonomously, or it can functionally synergize with AF1 or AF2. Autonomous AF3 function may explain the unexpected transactivating actions of antiprogestin-occupied hPR_B -- an issue of importance in hormone resistant breast cancers, and in tissue-specific agonist-like effects of hormone antagonists.

To complete Aim 2, the next steps are: to map AF3 in BUS by two methods: one is to create specific phosphorylation mutants of BUS at 5 *ser-pro* motifs and assess the functional state of this mutated BUS. We hope to deduce the role of phosphorylation in BUS transcriptonal activity by this method. The other is to create a series of random BUS mutants in order to knockout BUS function. We hope to precisely map AF3 by this method. These mutants will be assessed in a yeast expression system.

Additionally, we now will begin the studies outlined in Aim 3.

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